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The Effect of a Single Session of Intermittent Hypoxia on Erythropoietin and Oxygen-Carrying Capacity

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The Effect of a Single Session of Intermittent Hypoxia on Erythropoietin and Oxygen-Carrying Capacity

by

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Dedication

This document is dedicated to my parents, Matt and Adele. Your endless love, support, and encouragement has allowed me to pursue my passions in my educational journey.

Acknowledgements

Thank you to my advisor, mentor, and friend, Dr. Sophie Lalande. I am so grateful for your support, attention and invaluable advice and I could not have done this without you.

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Abstract

The Effect of a Single Session of Intermittent Hypoxia on Erythropoietin and Oxygen-Carrying Capacity

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Intermittent hypoxia, defined as alternating bouts of breathing hypoxic and normoxic air, has the potential to improve oxygen-carrying capacity through an erythropoietin-mediated increase in hemoglobin mass. The purpose of this study was to determine the effect of a single exposure of intermittent normobaric hypoxia on erythropoietin levels and hemoglobin mass in young healthy individuals. Nineteen healthy individuals (10 women and 9 men, age: 24 ± 4 years, height: 174 ± 11 cm, weight: 72.2 ± 12.2 kg) participated in the study. Participants were randomly assigned to an intermittent hypoxia group (Hyp, n = 10) or a placebo intermittent normoxia group (Norm, n = 9). Intermittent hypoxia consisted of five 4-minute hypoxic cycles at a targeted arterial oxygen saturation of 90% interspersed with 4-minute normoxic cycles. Air was made hypoxic by titrating nitrogen to a breathing circuit connected to a tank of compressed air. Nitrogen was not added to the breathing circuit in the intermittent normoxia condition. Pulmonary gas exchange, arterial oxygen saturation, and hemodynamics, using finger plethysmography, were continuously assessed during the intervention. Erythropoietin levels were measured before and two hours following the completion of the protocol. Hemoglobin mass was assessed using the carbon monoxide rebreathing technique the day before and seven days after exposure to intermittent hypoxia or normoxia. As anticipated, the intermittent hypoxia

group had a lower arterial oxygen saturation than the intermittent normoxia group during the intervention (Hyp: 89 ± vs. Norm: 98 ± 1%, $p < 0.01$), which was equivalent to a lower fraction of inspired oxygen (Hyp: 0.119 ± 0.008, Norm: 0.209 ± 0.001, $p < 0.01$). Erythropoietin levels did not significantly increase following exposure to intermittent hypoxia (Hyp: 8.2 ± 4.5 to 9.0 ± 4.8, Norm: 8.9 ± 1.7 to 11.1 ± 2.1 mU/ml, $p = 0.56$). Hemoglobin mass did not change following exposure to intermittent hypoxia (Hyp: 10.6 ± 1.4 to 10.2 ± 1.4, Norm: 9.7 ± 1.6 to 9.4 ± 1.5 g/kg, $p = 0.48$). Exposure to intermittent hypoxia did not affect mean arterial pressure (Hyp: 91 ± 6 to 90 ± 6, Norm: 93 ± 12 to 93 ± 12 mmHg, $p = 0.84$) or heart rate (Hyp: 68 ± 8 to 74 ± 9, Norm: 68 ± 8 to 68 ± 8 mmHg, $p = 0.27$). Respiratory rate, tidal volume, end-tidal CO_2 and total minute ventilation were not affected by intermittent hypoxia. Thus, a 40-minute session of intermittent hypoxia was not sufficient to elicit a rise in erythropoietin levels or hemoglobin mass in young healthy individuals. A longer exposure to intermittent hypoxia at a lower arterial oxygen saturation may be necessary to trigger an erythropoietin-mediated increase in hemoglobin mass in young healthy individuals.

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LITERATURE REVIEW

RELATIONSHIP BETWEEN HEMOGLOBIN MASS AND VO_2MAX

Maximal oxygen consumption (VO2max) represents a measure of cardiorespiratory fitness. According to the Fick equation, VO2 is the product of cardiac output and arterio-venous oxygen (a-vO2) difference:

$$VO_2 = Cardiac\ Output \times (a - vO_2\ difference)$$

and

$$a - vO_2\ difference = CaO_2 - CvO_2$$

where CaO2 represents arterial oxygen content and CvO2 represents venous oxygen content. Therefore, cardiac output, the amount of blood pumped by the heart per minute, and the difference in oxygen content between arterial and venous blood determine oxygen consumption. Arterial oxygen content itself is defined as:

$$CaO_2 = 1.34\ (ml\ O_2\ per\ gram\ Hb) \times Hb\ (g/L) \times SaO_2\ (\%)$$

where 1.34 represents the oxygen-binding capacity of hemoglobin indicating that 1 g of hemoglobin can bind 1.34 ml of oxygen, Hb represents hemoglobin concentration or the amount of hemoglobin in a volume of blood, the iron-containing protein within red blood cells that carries almost all the oxygen in the blood, and SaO2 represents arterial oxygen saturation, measured as the percentage of oxygen bound to hemoglobin. At sea level, the percentage of oxygen in the air is 20.9%, which corresponds to an arterial oxygen saturation between 96-99% in young healthy individuals (Pittman 2011).

Hemoglobin mass represents the absolute mass of circulating hemoglobin in the body. Because the transport of oxygen from the lungs to the exercising muscles is achieved through the binding of oxygen to hemoglobin, a greater hemoglobin mass results in an increase in oxygen-carrying capacity (Schmidt and Prommer 2010), and consequently a greater oxygen consumption, as shown by the strong relationship between hemoglobin mass and VO2max (Figure 1). Interventions that increase hemoglobin mass should, therefore, result in improved oxygen-carrying capacity and VO2max.

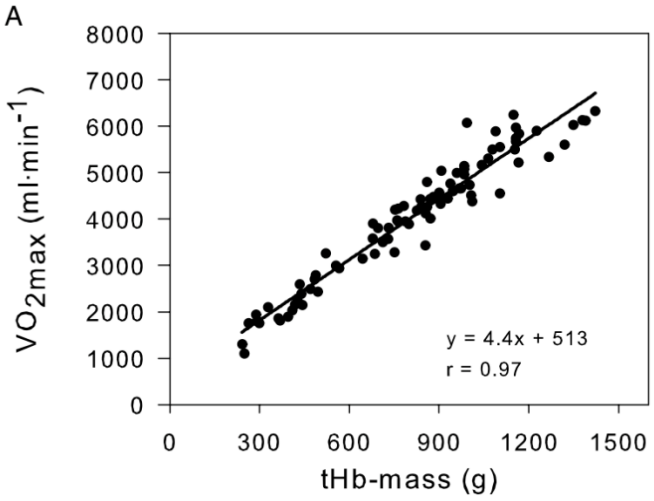

Figure 1. Relationship between absolute maximal oxygen uptake (VO_2max) and absolute total hemoglobin mass (tHb-mass) (Schmidt and Prommer 2010). Used with permission from Wolters Kluwer Heath, Inc.

Effect of hypoxia on erythropoietin and hematological variables

Exposure to low levels of oxygen, or hypoxia, stimulates the release of erythropoietin, the peptide hormone regulating the production of red blood cells, in order

to restore oxygen supply to the tissues. These exposures to low levels of oxygen can be achieved using hypobaric chambers or by breathing hypoxic gas mixtures under normobaric conditions (Ploszczyca et al. 2018). Following a rise in erythropoietin levels, it takes 5-6 days for a newly created reticulocyte to mature into a red blood cell and, consequently, observe an increased hemoglobin mass (Jelkmann 2011). Few studies (Eckardt et al. 1989, Knaupp et al. 1992, Klausen et al. 1996, Levine and Stray-Gundersen 1997, Rodriguez et al. 2000, Gore et al. 2006, Schmidt and Prommer 2010, Turner et al. 2017) have investigated the effects of short-term hypoxia on erythropoietin levels and hemoglobin mass.

Single exposure to continuous hypoxia

Few studies aimed to determine the hypoxic exposure duration needed to trigger an increase in erythropoietin levels. Single exposures to normobaric hypoxia at an oxygen level of 10.5% lasting 5 or 60 minutes were not sufficient to increase plasma erythropoietin levels in young healthy men (Knaupp et al. 1992). However, exposure to hypoxia for 120 minutes yielded a 50% increase in plasma erythropoietin levels (Knaupp et al. 1992). Similarly, 2 continuous hours of normobaric hypoxia at ~13.55 % oxygen and 2 continuous hours of hypocapnic hypoxia at 10% oxygen increased erythropoietin levels by 22% and 28%, respectively, in young healthy men (Klausen et al. 1996, Turner et al. 2017). A single session of 90 minutes of hypoxia triggered a 55% rise in erythropoietin levels measured 3 hours following the end of the hypoxic exposure in healthy men (Rodriguez et al. 2000). In addition, erythropoietin levels significantly increased by 33% following 90 minutes of

acute hypoxia exposure at a partial pressure of oxygen of 92 mmHg (Schmidt et al. 1991). In all cases, increases in erythropoietin levels were observed to peak approximately 2-3 hours after termination of these continuous hypoxic exposures (Knaupp et al. 1992, Klausen et al. 1996, Rodriguez et al. 2000, Turner et al. 2017). None of these studies assessed red blood cell count or hemoglobin mass.

Repeated exposures to continuous hypoxia

Red blood cell count significantly increased following exposure to 90 minutes of hypoxia 3 times a week over 3 weeks in healthy men (Rodriguez et al. 2000). The increase in red blood count was significant after 2 weeks of exposure, and reached its highest value in the third week (Rodriguez et al. 2000). Although measures of erythropoietin levels were not performed, similar results were obtained following exposures to 3-5 hours of continuous hypoxia for 9 consecutive days, with an approximately 10% increase in red blood cell count in young healthy individuals (Rodriguez et al. 1999). On the other hand, while erythropoietin levels increased by 50% 3 hours following exposure to hypoxia, there was no increase in red blood cell volume or hemoglobin mass in collegiate runners and swimmers after 4 weeks of hypobaric hypoxia exposure (3 hours/day, 5 days/week) (Gore et al. 2006). Despite the elevated erythropoietin levels, the authors proposed that the dose of hypoxia, both oxygen levels and duration, was insufficient to stimulate erythropoiesis (Gore et al. 2006). Neocytolysis, the death of red blood cells which can occur after returning to normal barometric pressure, could explain the inability to trigger a sustained increase in red blood cell volume (Gore et al. 2006).

Intermittent hypoxia

Intermittent hypoxia, defined as repeated short episodes of breathing hypoxic air interspersed with periods of breathing normoxic air, can also stimulate an increase in erythropoietin levels and red blood cell production (Knaupp et al. 1992, Burtscher et al. 2004, Burtscher et al. 2009). Knaupp *et al.* (1992) demonstrated that 240 minutes of intermittent hypoxia, alternating between 2.5 minutes of breathing oxygen levels of 10.5% and 1.5 minutes of normoxic air, significantly increased erythropoietin levels by 52% in young healthy men. On the other hand, Julian *et al.* (2004) used a protocol of 5:5 minute of hypoxia-to-normoxia ratio for 70 minutes, 5 times a week, with oxygen levels progressively decreasing from 12 to 10% over 4 weeks in elite athletes but failed to see any elevations in erythropoietin levels or hemoglobin concentrations. However, several methodological limitations could explain this lack of increase in erythropoietin levels and hemoglobin concentration. First, erythropoietin levels, which return to baseline levels 6 hours following a hypoxic exposure (Klausen et al. 1996), were measured at the same time each day (between 8 and 10 am) on the morning after participants were exposed to intermittent hypoxia, consequently preventing the detection of the rise in erythropoietin levels taken place in the first few hours following the hypoxic exposure. Second, the authors measured hemoglobin concentration, and not hemoglobin mass, which depends on plasma volume and is therefore greatly influenced by hydration status (Stray-Gundersen et al. 1992). Moreover, hemoglobin concentration correlates only to a modest extent with red blood cell mass (Schmidt and Prommer 2010). Hemoglobin mass is therefore a better marker of oxygen-carrying capacity (Schmidt and Prommer 2010). Third, elite endurance-trained athletes possess 40-50 % higher hemoglobin mass compared with sedentary individuals (Heinicke et al. 2001, Schmidt and Prommer 2010). Therefore, sedentary

individuals may be more likely to experience an increase in red blood cell production stimulated by a hypoxia-induced elevation in erythropoietin levels.

Beneficial effects of intermittent hypoxia have been observed in untrained populations. Burtscher *et al.* (2004) used an intermittent protocol of 3 to 5 hypoxic bouts with oxygen levels progressively decreasing from 14 to 10% and exposure duration progressively increasing from 3 to 5 minute over a period of 3 weeks in elderly men with and without coronary artery disease. This intermittent hypoxia protocol significantly increased red blood cell count by 3.9%, indicating an improved oxygen-carrying capacity. This increase in red blood cell count correlated to an increased VO_2peak in these individuals. A similar 3-week protocol of intermittent hypoxia at progressively declining oxygen levels of 15 to 12% increased total hemoglobin mass in patients at risk for or with mild, chronic obstructive pulmonary disease (Burtscher et al. 2009). Thus, exposure to intermittent hypoxia was reported to increase erythropoietin levels in young healthy individuals, and to increase red blood cell count and hemoglobin mass in clinical populations. It remains to be determined whether a single exposure to intermittent hypoxia increases hemoglobin mass in young healthy individuals.

Significance

The effect of a single exposure of intermittent hypoxia on both erythropoietin levels and hemoglobin mass remains equivocal in young, recreationally active healthy individuals. The aim of the present study was to determine whether a short 40-minute session of intermittent hypoxia, alternating 4 minutes of hypoxia at an arterial oxygen saturation of 90% with 4 minutes of normoxia, increases erythropoietin levels and

hemoglobin mass in young, recreationally active individuals. We hypothesized that intermittent hypoxia would increase erythropoietin levels and hemoglobin mass in young, recreationally active individuals.

MANUSCRIPT

INTRODUCTION

Maximal oxygen consumption (VO_2max) represents the ability of the cardiovascular system to transport and use oxygen during maximal exercise. Oxygen transport from the lungs to the exercising muscles is achieved through the binding of oxygen to hemoglobin, a protein located in the red blood cell. The total mass of hemoglobin in the blood strongly correlates to VO_2max (Schmidt and Prommer 2010). Therefore, interventions that increase hemoglobin mass should improve oxygen-carrying capacity and, consequently, VO_2max.

Exposure to low levels of oxygen, or hypoxia, stimulates the release of erythropoietin, the peptide hormone regulating the production of red blood cells. Following a rise in erythropoietin levels, it takes 5-6 days for a newly created reticulocyte to mature into a red blood cell, and to observe an increase in hemoglobin mass (Jelkmann 2011). Short exposures to continuous hypoxia lasting 90 min to 5.5 hours stimulate an increase in erythropoietin (Eckardt et al. 1989, Knaupp et al. 1992, Klausen et al. 1996, Rodriguez et al. 2000, Gore et al. 2006, Turner et al. 2017). No increase in erythropoietin levels was found after 5- and 60-min exposures to acute hypoxia at a fraction of inspired oxygen of 0.105, however, a 50% increase in erythropoietin levels was seen following a 120-min hypoxic exposure (Knaupp et al. 1992). Others found similar increases in erythropoietin after 2 hours of hypoxia at a fraction of inspired oxygen of 0.135, 0.125 and 0.115 (Turner et al. 2017). While a single exposure of hypoxia stimulates an increase in erythropoietin levels, hemoglobin mass was not measured in these studies. Red blood cell volume increased as a result of repeated 90 min sessions of hypoxia, 3 days a week for 3 weeks

(Rodriguez et al. 1999, Rodriguez et al. 2000). However, 4 weeks of hypoxia for 3 hours a day, 5 days a week at an altitude of 4,000-5,000 m increased erythropoietin levels but not red blood cell production in well-trained athletes (Gore et al. 2006).

Intermittent hypoxia consists of repeated short episodes of breathing hypoxic air interspersed with periods of breathing normoxic air. The proposed beneficial effect of intermittent hypoxia is that repeated stimulation of erythropoietin production would shorten the needed hypoxic exposure duration in order to increase oxygen carrying capacity (Rodriguez et al. 1999, Julian et al. 2004). Intermittent hypoxia lasting 240 minutes, alternating 2.5-minutes of hypoxia at 10.5% oxygen with 1.5-minutes of normoxia, significantly increased erythropoietin levels in young healthy men (Knaupp et al. 1992). Shorter repeated sessions of intermittent hypoxia consisting of hypoxic bouts of 3-5 min with 3 min normoxic periods at a progressively decreasing fraction of inspired oxygen from 0.15 to 0.10, 5 times a week for 3 weeks, increased hemoglobin mass in men with and without coronary artery disease and individuals with mild chronic obstructive pulmonary disease (Burtscher et al. 2004, Burtscher et al. 2009). However, it remains unknown whether a single session of intermittent hypoxia increases hemoglobin mass. Thus, the objective of the present study was to evaluate if a short single session of intermittent hypoxia is sufficient to increase erythropoietin and hemoglobin mass in healthy individuals.

METHODS

Twenty young healthy individuals were recruited for the study, but carboxyhemoglobin levels could not be measured in one participant and was removed from the study leaving a total of nineteen participants (10 women) who completed the study. All participants provided informed written consent for participating in the study, which was approved by the Institutional Review Board of the University of Texas at Austin. Participants were excluded from the research project if they had a resting blood pressure above 140/90 mmHg, smoked cigarettes, were pregnant, had a history of cardiovascular disease, diabetes or lung disease, or were taking medication affecting the cardiovascular system.

Study protocol

Participants visited the Clinical Exercise Physiology Laboratory on 3 occasions over a period of 8 days. Nineteen individuals were randomly assigned to an intermittent hypoxia group (Hyp, n = 10, 5 women) or a placebo normoxic group (Norm, n = 9, 5 women). During Visit 1 and 3, measures of hemoglobin mass, red blood cell volume, plasma volume, and total blood volume were performed. Visit 2 included measures of erythropoietin levels before and 2 hours following the completion of an intermittent hypoxia protocol or a placebo normoxic protocol. Visit 2 took place the day after Visit 1 and Visit 3 took place 7 days after Visit 2.

Intermittent hypoxia

The intermittent hypoxia protocol consisted of five 4-minutes hypoxic cycles (arterial oxygen saturation of 90%) interspersed with 4-minutes normoxic cycles (Figure 2) lasting a total of 40 minutes.

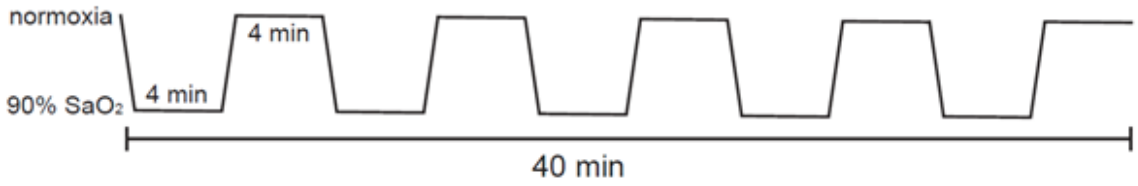

Figure 2: Intermittent hypoxia protocol

Participants inhaled hypoxic air through a mask connected to a 2-way non-rebreathing valve (Hans Rudolph, Inc, USA), which itself was connected to a 5-liter non-diffusing gas bag (Hans Rudolph, Inc, USA). The rebreathing bag was connected to a gas tank of compressed air. Air was made hypoxic by introducing nitrogen in the breathing circuit on the inspiratory port of the non-rebreathing valve. The flow of nitrogen was controlled to achieve an arterial oxygen saturation of 90%, as measured by pulse oximetry. Participants in the Norm group performed the same protocol, but nitrogen was not introduced in the breathing circuit. Gas exchange, hemodynamics, and arterial oxygen saturation were continuously measured during the intermittent hypoxia protocol and placebo normoxic protocol.

Hematological variables

Hemoglobin mass was determined using the optimized carbon monoxide rebreathing technique (Lalande et al. 2012). Upon arrival to the laboratory, participants laid down on a treatment table, and all measures were performed in this position. A blood sample was first drawn via venipuncture from the antecubital fossa of the arm to measure carboxyhemoglobin, hematocrit, and hemoglobin levels (ABL 80 FLEX OSM, Radiometer, Copenhagen, Denmark). Participants then rebreathed a body size-related dose of carbon monoxide for a period of 2 minutes, while keeping a normal breathing rate. Carboxyhemoglobin levels were measured again 10 minutes following the start of the carbon monoxide rebreathing. Hemoglobin mass was calculated from the measured change in carboxyhemoglobin levels induced by the carbon monoxide rebreathing. Red blood cell volume, plasma volume and blood volume were calculated from hemoglobin mass.

Hemodynamics

An arterial waveform obtained by finger plethysmography from the middle finger of the left hand was continuously recorded (NOVA, Finapres Medical Systems, Amsterdam, Netherlands). Brachial arterial blood pressure, heart rate, stroke volume, cardiac output and total peripheral resistance were derived from the arterial waveform, a method that has been validated against invasive measures (Wesseling et al. 1993). Arterial oxygen saturation was also continuously monitored by pulse oximetry (NOVA, Finapres Medical Systems, Amsterdam, Netherlands) throughout the intermittent hypoxic protocol

and placebo normoxic protocol. All data was recorded in LabChart (Powerlab, ADInstruments Inc., CO, USA) for later analysis.

Pulmonary gas exchange

Breath-by-breath measures of pulmonary gas exchange such as volume of oxygen and carbon dioxide, respiratory rate, tidal volume and minute ventilation were determined from a pneumotachometer (Ultima Cardio2, MGC Diagnostics, MN, USA) throughout the intermittent hypoxic protocol and placebo normoxic protocol. The pneumotachometer was mounted between the mask and the non-rebreathing valve of the breathing circuit.

Erythropoietin levels

Upon arrival to the laboratory on Visit 2, a blood sample was first drawn via venipuncture from the antecubital fossa of the arm for later analysis of erythropoietin levels. Another blood draw was performed 2 hours following the completion of the intermittent hypoxia protocol or the placebo normoxic protocol. Following collection, blood was centrifuged and serum aliquoted and stored at -80°Cfor subsequent analyses. Erythropoietin levels were determined using an enzyme-linked immunosorbent assay (Abcam, Cambridge, UK).

Data and statistical analysis

The last minute of each hypoxic cycle and normoxic was averaged in order to get the most stable values between all subjects. A one-way repeated measured analysis of variance was used to evaluate whether each cycle of intermittent hypoxia or intermittent normoxia triggered the same physiological responses. There was no difference in pulmonary gas exchange, hemodynamics and arterial oxygen saturation across hypoxic cycles, therefore, average values were calculated for each variable. Similarly, there was no difference in any variables across normoxic cycles, and average values were calculated for each variable. A one-way repeated-measures analysis of variance was used to evaluate the effect of conditions (Hyp and Norm) on participants' characteristics, pulmonary gas exchange, hemodynamics and arterial oxygen saturation. A two-way repeated measures analysis of variance was used to evaluate the effect of group (Hyp and Norm) and time (pre and post) on erythropoietin levels and hematological variables. When appropriate, post hoc analyses were performed using Tukey's test. Significance was set at $p \leq 0.05$. All values are reported as mean ± standard deviation.

RESULTS

Age, weight, height, hemoglobin concentration, hematocrit levels, resting blood pressure, heart rate and physical activity levels were not different between groups (Table 1). As anticipated, the Hyp group had a lower arterial oxygen saturation than the Norm group during the intervention (Table 2), which was equivalent to a lower fraction of inspired oxygen (Table 3). Exposure to intermittent hypoxia did not affect any hemodynamic or pulmonary gas exchange variables (Tables 2 and 3).

Erythropoietin levels did not change following exposure to intermittent hypoxia (Figure 3). Plasma volume did not change following exposure to intermittent hypoxia (Hyp: 3730 ± 583 to 3592 ± 506, Norm: 3151 ± 508 to 3044 ± 481 ml, $p = 0.48$). Hemoglobin mass did not change after intermittent hypoxia or normoxia (Hyp: 789 ± 212 to 760 ± 203, Norm: 683 ± 134 to 664 ± 137 grams, $p = 0.68$). Hemoglobin mass normalized to weight, red blood cell volume and blood volume did not change following intermittent hypoxia (Figure 4).

Table 1. Participants' characteristics

	Hypoxia	Normoxia
Age (years)	24 ± 2	24 ± 6
Weight (kg)	73.6 ± 13.3	70.7 ± 11.4
Height (cm)	176 ± 11	171 ± 10
Hemoglobin (g/dl)	13.9 ± 1.6	14.3 ± 1.3
Hematocrit (%)	42.8 ± 4.7	43.7 ± 3.9
Systolic blood pressure (mmHg)	115 ± 11	120 ± 16
Diastolic blood pressure (mmHg)	69 ± 8	72 ± 9
Heart rate (bpm)	65 ± 8	64 ± 7
Physical activity levels (hours/week)	4.8 ± 2.4	4.4 ± 2.6

Table 2. Hemodynamics during intermittent hypoxia or normoxia

	Hypoxia	Normoxia
Systolic blood pressure (mmHg)	126 ± 9	129 ± 16
Diastolic blood pressure (mmHg)	73 ± 7	75 ± 11
Heart rate (bpm)	74 ± 9	68 ± 8
Mean arterial pressure (mmHg)	90 ± 6	93 ± 12
Stroke volume (ml)	84 ± 20	82 ± 15
Cardiac output (L/min)	6.2 ± 1.5	5.6 ± 1.2
Total peripheral resistance (mmHg/L/min)	15.9 ± 4.2	17.7 ± 4.5
Arterial oxygen saturation (%)	88.5 ± 1.3 *	98.5 ± 1.0

* $p < 0.05$ between Hyp and Norm

Table 3. Pulmonary gas exchange during intermittent hypoxia or normoxia

	Hypoxia	Normoxia
Fraction of inspired oxygen (%)	11.9 ± 0.8 *	20.9 ± 0.1
End-tidal CO_2 (mmHg)	34.9 ± 2.8	36.8 ± 3.4
Ventilation (L/min)	7.9 ± 3.1	7.4 ± 2.3
Tidal volume (ml)	684 ± 334	515 ± 142
Respiratory rate (breaths/min)	14.0 ± 4.0	14.9 ± 3.0

* $p < 0.05$ between Hyp and Norm

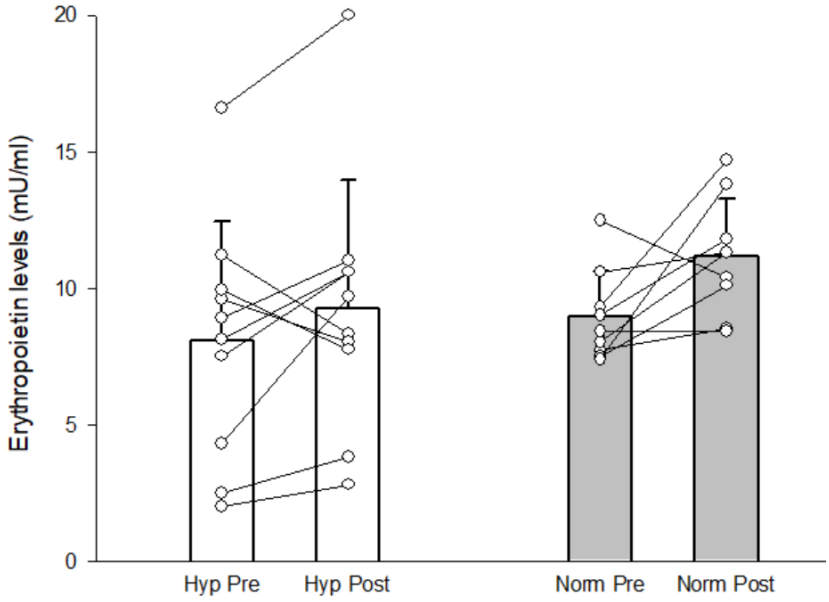

Figure 3. Erythropoietin levels before and 2 hours following the end of intermittent hypoxia and intermittent normoxia.

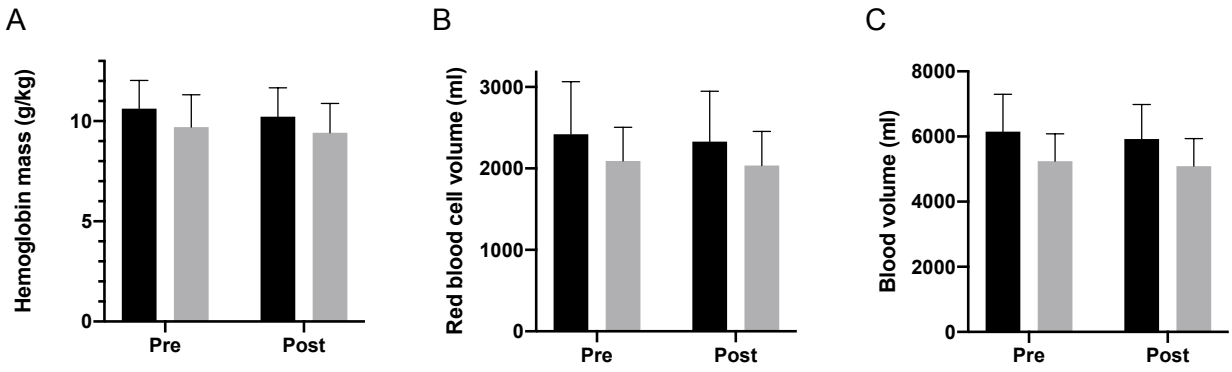

Figure 4. Hemoglobin mass (A), red blood cell volume (B) and blood volume (C) pre- and post-intermittent hypoxia (black bars) and intermittent normoxia (gray bars).

DISCUSSION

The purpose of this study was to determine whether a single session of intermittent hypoxia increases erythropoietin levels and hemoglobin mass in young healthy individuals. Contrary to our hypothesis, a 40-minute session of intermittent hypoxia was not sufficient to elicit a rise in erythropoietin levels or oxygen-carrying capacity in young healthy individuals.

Repeated exposures to intermittent hypoxia over several days and weeks leads to increased oxygen-carrying capacity (Rodriguez et al. 2000, Burtscher et al. 2004, Burtscher et al. 2009). Individuals with coronary artery disease showed an increase in red blood cells after 3 weeks of repeated sessions of intermittent hypoxia consisting of three to five hypoxic bouts at 14-10% oxygen, each lasting 3-5 min and separated by 3 min normoxic intervals (Burtscher et al. 2004). In addition, people at risk for or with mild chronic obstructive pulmonary disease performed a similar intermittent hypoxia protocol with a gradual decrease in oxygen from 15% to 12% and also demonstrated significant increases in hemoglobin mass after 3 weeks (Burtscher et al. 2009). Although erythropoietin levels were not measured in these studies, it is reasonable to assume that the hypoxia-induced increase in erythropoietin levels resulted in a greater red blood cell count. Although we used a similar intermittent hypoxia protocol to these previous studies (Burtscher et al. 2004, Julian et al. 2004, Burtscher et al. 2009), we did not see an increase in erythropoietin levels or hemoglobin mass in young healthy individuals. The timing of our erythropoietin measurements as well as the duration and severity of hypoxia possibly contributed to the observed lack of change in erythropoietin levels following exposure to intermittent hypoxia.

Timing of erythropoietin response to hypoxia

In the present study, erythropoietin levels did not change following a 40-minute exposure to intermittent hypoxia. Numerous studies reported that erythropoietin levels reach maximum levels 2 to 3 hours after the completion, or 4 to 6 hours following the onset, of a single session of hypoxia lasting between 90 minutes to 3 hours (Schmidt et al. 1991, Knaupp et al. 1992, Klausen et al. 1996, Rodriguez et al. 2000, Gore et al. 2006, Turner et al. 2017). Similar to these previous studies, we performed measurements of erythropoietin levels 2 hours following the end of intermittent hypoxia. However, due to our shorter intermittent hypoxia protocol, these measurements occurred approximately 3 hours following the onset of hypoxia, in comparison to previous studies observing peak levels between 4 and 6 hours after the onset of hypoxia. It is therefore possible that we did not detect the maximal increase in erythropoietin levels, and that erythropoietin levels were still rising following the exposure to intermittent hypoxia.

It is also possible that the time at which erythropoietin levels peaks varies between exposures to continuous vs. intermittent hypoxia. During an intermittent hypoxia protocol of 240 minutes, consisting of 2.5 minutes of hypoxia at oxygen levels of 10.5% and 1.5 minutes of nomoxia for a total hypoxic duration of 108 minutes, erythropoietin levels were significantly greater 6 hours after the onset of hypoxia. In comparison, peak levels of erythropoietin occurred 4 hours following exposure to 120 minutes of continuous hypoxia at oxygen levels of 10.5% (Knaupp et al. 1992). While the time spent under hypoxia was similar (108 vs. 120 minutes) and peak erythropoietin levels occurred 2 hours after completion of both protocols, peak levels from the onset of hypoxia occurred 2 hours apart (4 vs. 6 hours) between the continuous and intermittent protocols. Thus, an intermittent

protocol may delay the erythropoietin response to hypoxia, which possibly contributed to the observed lack of change in erythropoietin levels measured approximately 3 hours following our intermittent hypoxia protocol.

There is a gradual rise in erythropoietin levels throughout the day, with levels peaking during the evening and night hours, increasing by 60% from the lowest levels occurring a few hours after waking (Wide et al. 1989, Klausen et al. 1993, Klausen et al. 1996). All pre-intervention erythropoietin measurements occurred between 9 am and 12:30 pm. Post-intervention erythropoietin measurements were scheduled 2 hours following the end of intermittent hypoxia, and thus occurred between 12:15 and 2:37 pm. Consequently, all blood samples were obtained during the time period with the lowest erythropoietin levels. Therefore, it is unlikely that a large diurnal variation masked an increase in erythropoietin levels. Using each participant as its own control and performing all testing at the same time of day would have been beneficial in mitigating the effect of diurnal variation in erythropoietin levels.

Duration and severity of hypoxia

While it is possible that we missed the peak in erythropoietin levels, it is more likely that the short duration of our protocol better explains the lack of changes in erythropoietin levels. Previous findings suggest that a minimum hypoxic duration of 90 minutes is needed to elicit a significant increase in erythropoietin levels (Schmidt et al. 1991, Knaupp et al. 1992, Klausen et al. 1996, Gore et al. 2006, Turner et al. 2017). Knaupp *et al.* (1992) measured erythropoietin levels in response to 5, 60, and 120 minutes of continuous hypoxia

at oxygen levels of 10.5%, and after 240 minutes of intermittent hypoxia. While 5 and 60 minutes of continuous hypoxic was not sufficient to increase erythropoietin levels, 120 minutes of continuous hypoxia and 240 minutes of intermittent hypoxia caused a 50% increase in erythropoietin levels. Therefore, the total duration of hypoxic exposure (20 minutes) of our intermittent hypoxia protocol may have been too short to elicit changes in erythropoietin levels in young healthy individuals. Nevertheless, Burtscher *et al.* (2004, 2009) performed similar total hypoxic exposures lasting between 9 and 25 minutes, and repeated exposures to these short hypoxic durations were effective in increasing red blood cell volume and hemoglobin mass.

Our protocol targeted an arterial oxygen saturation of 90% which was equivalent to breathing 12% oxygen in young healthy individuals. However, many studies report significant erythropoietin level increases at lower arterial oxygen saturations, but not necessarily lower oxygen levels. While 2 hours of continuous hypoxia at oxygen levels of 11.5% and 12.5%, equivalent to arterial oxygen saturations of 78% and 83%, increased erythropoietin levels, erythropoietin levels were not significantly elevated at an oxygen level of 13.5% which corresponded to an oxygen saturation of 87% (Turner et al. 2017). According to oxygen levels, these findings imply that the severity of our protocol was adequate to trigger an increase in erythropoietin levels, however, the resulting arterial oxygen saturations suggest that the 90% oxygen saturation used in this study may not have been severe enough to stimulate erythropoietin release in young healthy individuals.

Individual variability in the erythropoietin response to hypoxia

Previous studies reported a large variability in the erythropoietin response to hypoxic exposure (Eckardt et al. 1989, Turner et al. 2017). A potential contributor to this individual variability is the use of fixed oxygen levels to induce hypoxia, which results in varying levels of hypoxemia as observed through different arterial oxygen saturations across participants. To attenuate the variability in individual responses to hypoxia, we customized the severity of hypoxia by titrating the flow of nitrogen into the breathing circuit to achieve a targeted arterial oxygen saturation with variable oxygen levels. Therefore, we believe that we were able to minimize the individual variability in the erythropoietin response to intermittent hypoxia.

Pulmonary gas exchange and hemodynamics

The intermittent hypoxia protocol used in the present study resulted in an average arterial oxygen saturation of 89%, which was equivalent to oxygen levels of 11.9%. This hypoxic exposure did not significantly affect any measures of pulmonary gas exchange or hemodynamics in young healthy individuals. Increases in heart rate were observed when arterial oxygen saturations dropped below 80% (Klausen et al. 1996, Faulhaber et al. 2015, Turner et al. 2017). While exposure to acute hypoxia may lead to increases in blood pressure through β-adrenergic stimulation (Prabhakar et al. 2005, Burtscher et al. 2009, Mateika et al. 2015), our data support previous literature stating that intermittent hypoxia is safe under moderate levels of hypoxia (ranging between 9-14%) and hypoxic durations of less than 2 hours.

In conclusion, a 40-minute session of intermittent hypoxia at an arterial oxygen saturation of 90% was not sufficient to elicit a rise in erythropoietin levels or hemoglobin mass in young healthy men and women. It is possible that we did not detect a rise in erythropoietin levels due to a premature measure of erythropoietin levels. Repeated sessions and/or a longer exposure to intermittent hypoxia may be necessary to trigger an erythropoietin-mediated increase in hemoglobin mass in young healthy individuals.

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